TRANSFORMATION PROCESS

THIS INVENTION relates to a method for obtaining transformed or genetically modified plant seed. It also relates to a transformation composition.

According to one aspect of the invention there is provided a method for obtaining transformed or genetically modified plant seed, the method including contacting germinating plant seed with a wetting agent or surfactant and an *Agrobacterium* strain to transform or genetically modify the plant seed.

The wetting agent or surfactant may be any suitable wetting agent or surfactant which facilitates or enhances penetration and transformation of germinating plant seed by the *Agrobacterium* strain. As hereinafter used, the term "wetting agent" includes surfactants with wetting properties. The wetting agent may be a non-oil based wetting agent, and may include a polyether polymethyl siloxane copolymer. One example of a suitable wetting agent is Break-Thru (available from Goldschmidt Chemical Corporation in Hopewell, USA). It is believed that the active component of Break-Thru is polyether polymethyl siloxane copolymer, Break-Thru being a non-oil wetting agent.

The *Agrobacterium* strain and the wetting agent may be in the form of an admixture or suspension. The wetting agent and the *Agrobacterium* strain may be present in the admixture in a mass ratio of the wetting agent: *Agrobacterium* strain of between 1:99 and 1:10000, e.g. 1:1000.

The germinating plant seed may be subjected to vacuum infiltration while they are being contacted with the wetting agent and the *Agrobacterium* strain. The germinating plant seed may be subjected to vacuum infiltration for a period of between 5 minutes and 40 minutes, e.g. 20 minutes, at a pressure of between 150 Pa (a) and 750 Pa (a), e.g. 585 Pa (a). The vacuum infiltration may be carried out at a temperature of between 15 °C and 35 °C, e.g. 25 °C.

The germinating plant seed may then be contacted with the admixture for a period of between 2 hours and 48 hours, e.g. 24 hours, at a temperature of between 15 °C and 35 °C, e.g. room or ambient temperature.

The *Agrobacterium* strain may be any suitable strain such as *Agrobacterium tumefaciens*, for example, *Agrobacterium tumefaciens* strain LBA4404 deposited at Centraalbureae voor Schimmel-cultures (CBS) in the Netherlands under No. CBS 191.83 on 24 February 1983.

The germinating plant seed may be transformed by the introduction of foreign DNA via the *Agrobacterium* strain. Thus, the germinating plant seed may be transformed by exposing or contacting the germinating plant seed with a culture of *Agrobacterium*, said *Agrobacterium* strain being transformation competent and including a construct comprising a foreign gene, the foreign gene including appropriate regulatory sequences so as to be expressed in the cells of a plant which is cultivated from the transformed or genetically modified plant seed.

The foreign gene may be any suitable gene, such as a foreign gene which confers disease resistance and/or drought resistance to the plant which is cultivated from the transformed or genetically modified plant seed.

The *Agrobacterium* strain may include a suitable plasmid to facilitate transformation of the plant seed.

The plasmid may include a vector, such as vector pBI121.

The method may include inducing further growth of the transformed plant seed and selecting for a transformant in the presence of a selecting agent. The *Agrobacterium* strain may include a plasmid which includes both said foreign gene and a selection agent resistance gene, the selection agent resistance gene also including appropriate regulatory sequences so as to be expressed in the cells of the plant which is cultivated from the transformed or genetically modified plant seed.

The selection agent resistance gene may code for antibiotic resistance, thus imparting resistance to an antibiotic selection agent to the plant which is cultivated from the transformed or genetically modified plant seed. The antibiotic selection agent may be selected from the group consisting of at least one of kanamycin and rifampicin, and the selection agent resistance gene may be a GUS-intron gene.

It will be appreciated that any suitable plant seed may be transformed using the method as herein described. The plant seed may be from the family *Leguminoseae* or any other dicotyledonous plant, for example, soybean or lupin seed. If soybean seed is used, the soybean seed may be allowed to germinate until it has a small plumule, easily removable seed coat and cotyledons which are not appressed against each other before the germinating soybean seed is contacted with the wetting agent and the *Agrobacterium* strain. If lupin seed

is used, the lupin seed may be allowed to germinate until the plumule is between 10 - 20 mm in size, before the germinating lupin seed is contacted with the wetting agent and the *Agrobacterium* strain.

The method may include germinating plant seed at a temperature of between 22 °C and 32 °C, e.g. about 29 °C, for a period of between 2 days and 5 days, e.g. about 4 days before contacting the germinating plant seed with the wetting agent and the *Agrobacterium* strain.

The invention extends to a transformed or genetically modified plant seed produced by the method as herein before described, to a transformed or genetically modified plant cultivated from said plant seed, to a plant seed produced by said transformed or genetically modified plant, and to a plant which is the progeny of said transformed or genetically modified plant.

The transformed or genetically modified plant may comprise cells which comprise in their genome at least one preselected foreign gene which produces a foreign cellular product encoded by the foreign gene. The foreign gene may code for at least one of disease resistance and drought resistance.

According to another aspect of the invention, there is provided a transformation composition which includes, in admixture, an *Agrobacterium* strain and a wetting agent.

The wetting agent may be a non-oil based wetting agent. The wetting agent may include a polyether polymethyl siloxane copolymer, and the *Agrobacterium* strain may be *Agrobacterium tumefaciens.*

The wetting agent and the *Agrobacterium* strain may be present in a mass ratio of the wetting agent: *Agrobacterium* strain of between 1:99 and 1:10000, e.g. 1:1000.

The invention will now be described by way of non-limiting example, with reference to the following Figures and examples of methods of transforming plant seed, in accordance with the invention.

Figure 1A shows X-GLUC histochemical localization of GUS enzyme activity in GUS transformed soybean cultivar Carnia 2233 leaf tissue;

Figure 1B shows X-GLUC histochemical localization of GUS enzyme activity in GUS transformed soybean cultivar Carnia 2233 root tissue;

Figure 2 shows X-GLUC histochemical localization of GUS enzyme activity in GUS-INT transformed soybean cultivar Carnia 2233 stomata;

Figure 3 shows the effect of mannitol stress on proline synthesis in third generation transformed soybean cultivar Carnia 2233 with an antisense P5CR construct.

Figure 4 shows a woodenbox screening of control soybean cultivar Carnia 2233 (without *Arabidopsis* P5CR gene) compared to transformed soybean cultivar Carnia 2233 (containing P5CR gene in antisense orientation);

Figure 5A shows the effect of higher copy number of P5CR gene on chlorophyll fluorescence in transformed soybean cultivar Ibis plants (normalised normal plant = NN; normalised transformant antisense orientation = NT AS);

Figure 5B shows the effect of higher or lower copy number of P5CR gene on chlorophyll fluorescence in transformed Ibis plants (normalised transformed antisense = NT AS; normalised transformed sense = NT S; normalised normal plant = NN); and

Figure 6 shows transformed lupin leaves of initial transformed seed, together with first generation seed compared to control plants leaves and seeds.

EXAMPLE 1

Agrobacterium tumefaciens strain LBA4404 containing a CaMV 35S GUS gene (pBI121) was cultured at 27°C in 100 ml Luria-Bertani broth (LB) pH 7.00 supplemented with 150μg/ml rifampicin and 100μg/ml kanamycin until an

absorbence of A_{600} = 0.5 was obtained. 0.01 mg/ml Acetosyringone was added to the *Agrobacterium tumefaciens* culture approximately 24 hours before transformation of plant seeds was carried out. The *Agrobacterium tumefaciens* culture was centrifuged at 10000 rpm for 20 minutes at a temperature of 10°C. Flocculation was avoided or inhibited by dilution of the *Agrobacterium tumefaciens* culture with distilled water to obtain a ratio of *Agrobacterium tumefaciens* : distilled water of 1:4. 0.1% Break-Thru (a wetting agent) (obtainable from Goldschmidt Chemical Corporation was added to the diluted *Agrobacterium tumefaciens* culture.

Soybean seed (Carnia 2233) was sterilised for 5 minutes in 3.5% (v/v) NaOCl, and then washed in sterile water before being germinated on sterile 0.8% water agar at a temperature of 29°C for a period of 2 to 5 days. The germinating soybean seeds were then sorted and soybean seeds having a small plumule, easily removable seed coat and cotyledons which were not appressed against each other, were selected. The selected germinating or germinated soybean seeds were then contacted with the *Agrobacterium*/ wetting agent suspension and vacuum infiltrated for 20 minutes under a pressure of 78 millitorr. After vacuum infiltration, the germinating soybean seeds were then incubated for a further period of 24 hours in contact with the *Agrobacterium*/wetting agent suspension solution at room or ambient temperature to obtain transformed soybean seeds. The transformed soybean seeds were then planted in a soil mixture comprising soil, sand, vermiculite (5:5:3) and grown in a greenhouse to obtain transformed soybean plants. Percentage success rate of transformation was determined by detecting GUS-gene activity using a fluorometric and a histochemical assay.

When the transformed plants had developed a second set of leaves, a leaf from the main apex was tested for expression of the GUS gene. A fluorometric GUS assay (Jefferson, R.A., Kavanagh, T.A. & Bevan, M.W. 1987). GUS fusion ß - glucuronidase as a sensitive and versatile gene fusion marker in

higher plants. *EMBO* J. 6 (13): 3901-3907 was used for screening of all explants for the expression of glucuronidase enzyme. 100 µl sodium phosphate buffer was pipetted into the wells of a microtiter plate. The assay buffer contained 50 mM $NaPO_4$ (pH7.00), 10mM EDTA, 0.1% (v/v) Triton X-100, 10mM mercapto ethanol and 2 M methyl umbilliferyl glucuronide (Sigma). Small pieces of plant tissue were crushed in this buffer and incubated overnight at a temperature of 37°C in the dark. Reactions were visualised on a long wave length UV light box. A histochemical assay was also performed in testing putative transformants. Plant tissue, which tested positive in the fluorescence assay, was incubated overnight at a temperature of 37°C in the dark in a histochemical staining solution. The staining solution contained 50mM $NaPO_4$ (pH7.00), 0.1% (v/v) Triton X-100, 1.04 mM X-Gluc and 0.5% (v/v) DMSO. The plant tissue was subsequently washed in FAA for 10 minutes, followed with a wash in 50% ethanol. The tissue was dehydrated with 100% ethanol and hydrated slowly up to 100% H_2O.

Transformed soybean plants (TO = plants obtained from initial transformed seed; T1, T2 and T3 = plants obtained from self pollinated, first to third generation, transformed plants) were tested for GUS activity and positive results were obtained indicating transformation. GUS expression patterns of soybean cultivar Carnia 2233 transformed with the CaMV 35S gene, were thus observed in leaf (Figure 1A) and root (Figure 1B) tissue of the plant. Plants tested positive for the GUS gene up to the third generation.

To verify the insertion of the GUS gene in the plant genome, molecular analysis, i.e. PCR reaction as well as a Southern blot procedure (Maniatis, T. Fritsch, E.F. & Sambrook, J., 1982. *Molecular Cloning : A Laboratory Manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, H.Y.), were conducted with GUS and NPTII specific primers. PCR and Southern blot observations indicated the presence of the GUS gene in the soybean genome. Plants tested positive up to the third transformant generation.

EXAMPLE 2

In another embodiment of the invention using essentially the same methods and techniques as described in Example 1, soybean seed (Carnia 2233) was transformed using an *Agrobacterium* strain LBA4404 containing a p35S GUS INT gene. It will be appreciated that a GUS-intron gene has the ability to discriminate between prokaryotic organisms such as *Agrobacterium tumefaciens* and eukaryotic organisms such as plants. Only plant tissue containing the GUS-INT gene turns blue in colour in association with a histochemical assay. Any *Agrobacterium* possibly still present in the plant tissue does not turn blue in colour. Figure 2 shows transformed plant cells which have been stained with X-GLUC, indicating that the soybean seed was transformed with the GUS-intron gene and that the GUS activity does not arise from endogenous *Agrobacteria*.

EXAMPLE 3

In a further embodiment of the invention using essentially the same methods and techniques described above for Example 1, soybean seed (Carnia 2233) was transformed with an antisense construct of a proline gene (L-D^1-pyrroline-5-carboxylate reductase = P5CR). The proline gene P5CR was obtained from N. Verbruggen, Laboratory of Genetics, University of Gent, Belgium. The P5CR gene was cloned in antisense orientation into plasmid HB101pMA445, containing a heat inducible promoter which was subsequently triparental mated to A*grobacterium tumefaciens* (Armitage, P. 1988. Transformation of dicotyl plant cells using the Ti plasmid of *Agrobacterium tumefaciens* and Ri plasmid of *A. rhizogenes*. In: Plant Genetic Engineering and Gene Expression: A laboratory Manual. Draper, J., Scott, R., Armitage, P. and Welden, R. (Eds). Blackwell Scientific Publications, Oxford. pp69-160). The construct included a kanamycin resistant gene which can be used in screening of transformants.

Putative transformed seed (with the P5CR gene) were tested for the presence of kanamycin resistance to indicate transformation. Third generation transformed soybean seed and untransformed soybean seed were tested for germination viability on agar plates supplemented with kanamycin. It was noted that all the seeds germinated on agar plates with 0mg/ℓ kanamycin (see Table 1 below). It was noted that as the concentration of kanamycin in the agar plates was increased, the percentage of germinating plants decreased. Some of the seed initially started to germinate for a short period before dying off and some of the germinating plants showed deformities. At the highest concentration of kanamycin in the agar plates, none of the untransformed soybean seeds germinated whereas in contrast 37.5% of the transformed soybean seeds were able to germinate successfully. The plants which germinated successfully were planted in a greenhouse and the results indicated that the transformed plants which germinated were most probably transformed with the P5CR gene.

Table 1: Germination of transformed soybean seed and untransformed control soybean seed on kanamycin supplemented agar plates

	0 mg/ℓ km	25 mg/ℓ km	35 mg/ℓ km	50mg/ℓ km
Untransformed soybean seed	100%	0%	0%	0%
Transformed soybean seed	100%	80%	75%	37.5%

km - kanamycin

The untransformed control plants and the transformed plants were subjected to a variety of tests including different physiological techniques e.g. proline accumulation and anatomical techniques e.g. woodenbox screening to compare the untransformed control plants with the transformed plants under drought and osmotic stress.

Inactivation of the P5CR gene resulted in decreased proline synthesis. The application of a mannitol stress test resulted in the untransformed control plants showing a significant increase in proline concentration whereas the antisense transformed plants displayed a significant decrease in proline concentration, indicating that the P5CR gene had been inactivated in the transformed plants and the transformed plants were unable to synthesise proline in response to the osmotic stress test.

The results of the tests indicate that antisense transformed plants were more drought sensitive than untransformed control plants, which indicates an underexpression of the P5CR gene, as a result of the antisense construct.

Control plants and fourth generation antisense transformed plants were subjected to a woodenbox experiment. Seed was planted in a box and watered until all plants reached the six leaf stage. At this stage the promoter was activated and drought stress was applied. The control plants survived a six day drought stress significantly more than most of the antisense transformed plants which died. The results indicate that soybean plants were successfully transformed with the antisense construct, as the transformed plants were unable to survive a drought stress test with a lower copy number of the proline gene (see Figure 4).

EXAMPLE 4

In order to test the reproducibility of the transformation method further, another soybean cultivar Ibis was transformed with a P5CR gene in sense and antisense direction. Essentially the same methods and techniques described above for Example 1 were used to transform the soybean cultivar Ibis with the P5CR gene. PCR demonstrated that transformation of the Ibis cultivar was successful. Sense and antisense transformants were evaluated physiologically to confirm transformation as well as the effect of the two different constructs on

Ibis. Detection of Ibis transformants was conducted using a chlorophyll fluorescence test. Changes in the photo system II activity of photosynthesis during drought stress can be analysed by measuring changes in different parameters of chlorophyll fluorescence.

The results of the chlorophyll fluorescence test are shown graphically in Figures 5A and 5B, in which the following abbreviations are used:

yO	-	A measurement of the ability of a trapped excition to move an electron into electron transport.
ABS/RC	-	The absorption flux of photons per photosystem II (PSII) reaction centre (RC).
ABS/CSm	-	The absorption flux of photons per PSII cross section (CS).
TRo/RC	-	The rate at which an excition is trapped by the RC.
ETo/RC	-	The electron transport per RC.
DIo/RC	-	The energy flux, which is wasted per RC as heat or transfer to other systems.
RC/CSo	-	Reaction centre per cross section.
RC/CSm	-	An indication of the number of active RCAEs (density) per CS.
TRo/CSm	-	The rate at which an exciton is trapped by the CS.
ETo/CSm	-	The electron transport per CS.
DIo/CSm	-	The energy flux, which is wasted per CSm as heat or transfer to other systems (Strasser B.J and Strasser R.J. 1995. Measuring fast fluorescence transients to address environmental questions: the JIP test. In: Photosynthesis: from light to biosphere (P.Mathis. Ed.) Vol. 5: 977-980. Kluwer Academic Publishers, Dordrecht. ISBN 0-7923-3862-6).

The antisense transformed plants (see Figure 5B) were subjected to stress tests which resulted in the antisense transformed plants increasing active

reaction centres, absorption, trapping and electron transport per cross section in compensating for stress imposed on the antisense transformed plants, whereas in contrast the sense transformed plants (see Figure 5A) when subjected to stress tests shut down certain reaction centres and there was a decrease in trapping, absorption and electron transport. The results indicated that sense transformed plants are better able to survive the stress tests as compared to the antisense transformed plants and control plants. Wasted energy per cross section was lower in the sense transformed plants than in the antisense transformed plants. The sense transformed plants contain a higher P5CR copy number and are therefore able to use energy more efficiently than the antisense transformed plants having a lower P5CR copy number. It appears that the sense transformed plants are more drought tolerant whereas the antisense transformed plants are more drought sensitive. These tests indicated that transformation of Ibis was successful.

EXAMPLE 5

In a further embodiment of the invention using essentially the same methods and techniques described above for Example 2, *Lupinus albus* seed (cultivar Esta) was transformed with *Agrobacterium* strain LBA4404 containing a p35S GUS INT gene. Figure 6 shows transformed lupin leaves of initial transformed seed, together with first generation seed compared to control plants, leaves and seeds. The transformed leaves and seed demonstrate blue colouring representing GUS-gene activity. This indicates that lupin was succesfully transformed using the method according to the invention and that the GUS INT gene was successfully transferred at least to the first generation.

SUMMARY

The methods in accordance with the invention resulted in a transformation success rate of approximately 35% of the soybean seeds. This

is a relatively high transformation success rate in that conventional techniques usually only have a transformation success rate of less than 5%. By inserting a foreign gene or genes into a plasmid in *Agrobacterium tumefaciens*, the soybean seed may be transformed with the foreign gene. The foreign gene is then included in the cells of a soybean plant which grows from the transformed soybean seed and may then be inherited by its progeny.

Advantages of the invention are that the methods are relatively easy to carry out and relatively inexpensive compared to conventional transformation procedures and techniques. As no tissue culture steps are used in the methods according to the invention, it is believed that there will be little or no loss of genetic traits which would usually occur as a result of somatic mutations. The methods in accordance with the invention can be used for transforming any suitable plant seed with genes of interest or agricultural usefulness, for example, drought resistant or disease resistant genes. The methods in accordance with the invention can also be used for producing transgenic plants of other species where routine tissue culture procedures have not yet been established.